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Comprehensive Census of Bacteria in Clean Rooms by Using DNA Microarray and Cloning Methods▽†

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A census of clean room surface-associated bacterial populations was derived from the results of both the cloning and sequencing of 16S rRNA genes and DNA microarray (PhyloChip) analyses. Samples from the Lockheed Martin Aeronautics Multiple Testing Facility (LMA-MTF), the Kennedy Space Center Payload Hazard and Servicing Facility (KSC-PHSF), and the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF) clean rooms were collected during the various assembly phases of the Phoenix and Mars Science Laboratory (MSL) spacecraft. Clone library-derived analyses detected a larger bacterial diversity prior to the arrival of spacecraft hardware in these clean room facilities. PhyloChip results were in agreement with this trend but also unveiled the presence of anywhere from 9- to 70-fold more bacterial taxa than cloning approaches. Among the facilities sampled, the JPL-SAF (MSL mission) housed a significantly less diverse bacterial population than either the LMA-MTF or KSC-PHSF (Phoenix mission). Bacterial taxa known to thrive in arid conditions were frequently detected in MSL-associated JPL-SAF samples, whereas proteobacterial lineages dominated Phoenix-associated KSC-PHSF samples. Comprehensive bacterial censuses, such as that reported here, will help space-faring nations preemptively identify contaminant biomatter that may compromise extraterrestrial life detection experiments. The robust nature and high sensitivity of DNA microarray technologies should prove beneficial to a wide range of scientific, electronic, homeland security, medical, and pharmaceutical applications and to any other ventures with a vested interest in monitoring and controlling contamination in exceptionally clean environments.

Planetary protection efforts work toward protecting (i) solar system bodies from contamination by terrestrial biological material (forward contamination), thus preserving opportunities for future scientific investigation, and (ii) the Earth from harmful contamination by materials returned from outer space (back contamination) (5). These approaches apply directly to the control and eradication of microorganisms present on the surfaces of spacecraft intended to land, orbit, fly by, or be in the vicinity of extraterrestrial bodies. Consequently, current planetary protection policies require that spacecraft be assembled and readied for launch in controlled clean room environments. To achieve these conditions and maintain compliance with good manufacturing practice regulations, robotic spacecraft components are assembled in ultraclean facilities. Much like facilities in the medical, pharmaceutical, and semiconductor sectors, National Aeronautics and Space Administration (NASA) spacecraft assembly clean rooms (SAC) are kept extremely clean and are maintained to the highest of industry standards (17). Filtered air circulation, controlled temperature and humidity, routine exposure to disinfectants and surfactants, and nutrient-limiting, oligotrophic conditions make it very challenging for microorganisms to persist in such environments, but these measures by no means eradicate biological contaminants entirely (18). Several investigations, both culture based and culture independent, have demonstrated that a variety of bacterial taxa are repeatedly isolated under clean room conditions (18, 24, 26; P. Vaishampayan, S. Osman, G. Andersen, and K. Venkateswaran, submitted for publication). However, despite a growing understanding of the diverse microbial populations present in SAC, predicting the true risk of any such microbes' compromising the findings of extraterrestrial life detection efforts remains a significant challenge (30). A better understanding of the distribution and frequency at which high-risk contaminant microbes are encountered on spacecraft surfaces would significantly aid in assessing the threat of forward contamination (33).

The purification of nucleic acids, subsequent PCR amplification, and shuttling of 16S ribosomal "fingerprint" genes from noncultivable microorganisms into genetically amenable lab strains of *Escherichia coli* have evolved into a gold standard of molecular means to elucidate the microbial diversity in a given sample. In theory, the cloning and sequencing of 16S ribosomal genes from each and every cell present, regardless of cultivability and inclusive of novel taxa, would result in a comprehensive survey of microbial communities on the surfaces of SAC and colocated spacecraft (24, 26). Unfortunately, the full-length sequencing of all 16S rRNA genes from environmental samples would be prohibitively expensive, making the ap-

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proach unfeasible for generating comprehensive phylogenetic profiles of complex microbial communities.

Attempting to infer population membership from clone libraries limited to hundreds or thousands of sequences has proven to be insufficient for detecting extremely low-abundance organisms. Recent analyses of phylogenetic DNA extracted from soil, water, and air revealed that laboriously derived clone libraries severely under-represent complex bacterial communities compared to very rapid (i.e., requiring only hours) DNA microarray approaches (1, 6, 11, 23, 36). One of the reasons for this is the high sensitivity of PhyloChip methodologies, which are able to detect organisms present in amounts below 10^{-4} abundance of the total sample (12). Numerous validation experiments using sequence-specific PCR have confirmed that taxa identified by the microarray were indeed present in the original environmental samples, despite their absence in corresponding clone libraries (3). This highlights the utility of the method compared to classical cloning. Although the analysis of each sample by the PhyloChip provides detailed information on microbial composition, the highly parallel and reproducible nature of this array allows tracking community dynamics over time and treatment. Even without prior sequence information, PhyloChip can identify specific microbial interactions that are key to particular changing environments.

A comprehensive census of the microbial communities on the surfaces in three NASA SAC supporting two distinct missions was conducted. To ensure that the maximum diversity of resident microbiota was uncovered, subsamples from each clean room surface sampling were subjected to both DNA microarray protocols and conventional cloning and sequencing of 16S rRNA genes. This study, to our knowledge the first of its kind, focused on comparing the microbial diversity profiles resulting from DNA microarray analyses and conventional cloning and sequencing of 16S rRNA genes arising from a variety of low-biomass surfaces.

MATERIALS AND METHODS

Sampling locations. Three different NASA clean rooms were sampled: the Lockheed Martin Aeronautics' Multiple Testing Facility (LMA-MTF), the Kennedy Space Center's Payload Hazardous Servicing Facility (KSC-PHSF), and the Jet Propulsion Laboratory's Spacecraft Assembly Facility (JPL-SAF). Each of the facilities examined was a certified class 100K (100,000 particles of >0.5 μm ft^{-3} air) or ISO 8 (3,520,000 particles of >0.5 μm m^{-3}) clean room. All spacecraft assembly facilities examined in this study were maintained with daily cleaning regimens consisting of the replacement of tacky mats, wiping of surfaces and support hardware fixtures, and vacuuming and mopping of floors with clean room-certified sanitizing agents (disinfectants, alcohol, and/or ultrapure water). Prior to entering the clean room, the staff was required to take appropriate actions to minimize the influx of particulate matter. Specific entry procedures varied depending on the certification level of the clean room and the presence or absence of mission hardware. All facilities utilized high efficiency particle air filters for continuous air filtration. Air was volumetrically exchanged a minimum of four times per hour, with positive pressure maintained at all times. For all three facilities, temperature, relative humidity, and airborne particle concentration were continuously monitored and recorded. Surface particulate matter, nonvolatile residue, and volatile hydrocarbons were monitored using conventional methods (27).

Sample collection. Samples were collected from the surfaces of various NASA SAC. Wet-surface sampling of the SAC floor (1 m^2) was performed using biological sampling kits (BiSKits; QuickSilver Analytics, Abingdon, MD) as previously described (4). Controls specific for each BiSKit were prepared in a class II biohood immediately prior to sampling. The manufacturer-provided buffer was brought to a volume of 30 ml using sterile phosphate-buffered saline (PBS) and was then added to the macrofoam sponge component of the BiSKit. The modified buffer was recovered from the sponge by screwing the sponge casing against the BiSKit cover several times, allowing sample to be collected into the attached sample bottle. A 15-ml portion of buffer was removed from the sample bottle and stored in 50-ml centrifuge tubes to serve as a sample control. The remaining modified buffer was used for sample collection. Once the PBS had adequately absorbed to the macrofoam sponge, the sampler was unscrewed from the module and was traversed about the surface area of interest (ca. 1 m^2), first in a horizontal fashion, then vertically, and finally in a diagonal sweeping pattern. Immediately following the collection of sample from a surface, the macrofoam sponge sampler was forcefully screwed back into the BiSKit module so as to squeeze as much sample as possible from the sponge into the collection tube. The module and attached collection tube were then transported in a sealed bag back to the laboratory, where they were further processed in a biohood. Overall, 10 distinct sampling events collected a total of 107 samples. The date of collection, functional relevance of the facility, and other sample characteristics are given in Table 1.

Concentration of biomolecules. Previous studies have demonstrated that SAC samples are seldom laden with levels of microbial biomass able to yield PCR-amplifiable DNA following extraction regimes; we therefore opted to pool several samples (24–26). All samples (~100 ml to 300 ml) were aseptically transferred to Amicon Ultra-15 centrifugal filter tubes (Ultracel-50 membrane, catalog no. UFC905096; Millipore, Jaffrey, NH), which were in turn placed within a Sorvall RC-5B refrigerated centrifuge (Thermo Scientific, Waltham, MA) and spun at 4,000 rpm for 10 min. Each filter unit has a molecular mass cutoff of 50 kDa, which facilitates the concentration of bacterial cells, spores, and exogenous nucleic acid fragments greater than 100 bp into a final volume of ca. 500 μl. This resulting volume was aseptically transferred to a sterile microcentrifuge tube. A comparable amount of sterile PBS was concentrated in a separate filter tube, serving as a negative control for each concentration/extraction.

Clone library construction and 16S rRNA sequencing. DNA was purified directly from each sample or pooled sample. Approximately 400 μl of each concentrated sample was subjected to bead beating and automated DNA extraction in an Autolyzer A-2 DNA extraction instrument (Axcyte Genomics, Menlo Park, CA), as demonstrated before (19). The total DNA extract (ca. 70 μl) arising from any given sample was then bifurcated for downstream DNA microarray and 16S rRNA cloning analyses. To generate PCR amplicons for subsequent cloning manipulations, bacterial 16S small subunit rRNA genes were PCR amplified with eubacterially biased primers B27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and B1512R (5′-AAG GAG GTG ATC CAN CCR CA-3′). Two distinct PCR regimes were employed in this study, gradient (see below) and conventional conditions, which were as follows: 1 min of denaturation at 95°C, 2 min of annealing at 55°C, and 3 min of elongation at 72°C for 35 cycles using a DNA Engine thermal cycler (MJ Research, Waltham, MA). After 10 min of incubation at 72°C, the amplification product was purified with a gel excision kit (Qiagen, Chatsworth, CA). PCR conditions as they relate to each sample are given in Table 1. Purified PCR amplicons were cloned into the pCR-4 TOPO vector, and recombinant plasmids were used to transform competent *E. coli* TOP10 cells via TA cloning protocols (Invitrogen, Carlsbad, CA), per the manufacturers' instruction. Approximately 1,000 clones each were analyzed and sequenced bidirectionally using M13F and M13R primers at Agencourt Biosciences Corp. (Beverly, MA).

Statistical, phylogenetic, and bioinformatics analyses. A program designed in-house (STITCH) was used to merge sequence pairs generating nearly full-length (~1,500 bp) 16S rRNA sequences (P. Vaishampayan et al., submitted). The phylogenetic relationship of clones was determined via comparison with quality-checked type strain 16S rRNA gene sequences (28) using the BLAST function in STITCH. Evolutionary trees were constructed using PAUP software (36). Rarefaction analysis (15) and coverage calculations (14) were applied to estimate the representation of the phylotypes in bacterial libraries. Operational taxonomic units (OTUs) were defined as clones sharing >97.5% sequence identity (21, 29, 34). The DOTUR program, version 1.53 (31), which considers the distance matrix in describing genetic distances between sequences and assigning them to OTUs, was used to analyze the data generated in this study. DOTUR uses the frequency at which each OTU is observed to construct rarefaction. The sequences were aligned by using ClustalW (20), and a Jukes-Cantor-corrected distance matrix was constructed by using the DNADIST program from PHYLIP (10). The rarefaction curve was produced by plotting the number of OTUs observed against the number of clones screened using DOTUR. The coverage of clone libraries was calculated (14) according to the following equation: $C = [1 - (n1/N)] \times 100$, where C is the homologous coverage, $n1$ is the number of OTUs appearing only once in the library, and N is the total number of clones examined.

PhyloChip PCR amplification. Bacterial 16S rRNA genes were amplified from pooled genomic DNA preparations from each sampling event using the primers

27f (5′-AGA GTT TGA TCC TGG CTC AG) and 1492r (GGT TAC CTT GTT ACG ACT T). PCR conditions were as follows: 1 cycle of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 48 to 57.5°C (various annealing temperatures), and 2 min at 72°C, with a final 10-min incubation at 72°C. To maximize observed diversity, four separate PCRs were performed for each sample using a gradient of annealing temperatures (48°C, 50.1°C, 54.4°C, and 57.5°C). Whenever possible, a total of 500 ng of amplified PCR fragments from each sampling event was used for phylogenetic microarray analysis.

PhyloChip processing, scanning, probe set scoring, and normalization. A detailed explanation of the processing of the PhyloChip assay has been previously published elsewhere (35). Briefly, the pooled PCR product from each sampling event was spiked with known amounts (5.02×10^8 to 7.29×10^{10} molecules) of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments (total, 200 ng). Fluorescence intensities from these controls were used as standards for normalization among samples. Target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining, as well as background subtraction, noise calculation and detection, and quantification criteria were performed as reported elsewhere (11). An OTU was considered present in the sample when 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction of >0.90).

Controls and lower detection limits of assays employed. Appropriate controls were used at each step of the sampling and analysis to ensure high-quality data. Liquid samples from unopened sample cartridges served as negative controls in all molecular assays. In the same manner, sterilized water, free of nucleic acids, served as a blank to monitor reagent cleanliness. Purified DNA from *Bacillus pumilus* ATCC 7061 was included in the PCR amplification protocols as a positive control. In this manner, samples containing inhibitory substances were monitored for false-negative results. None of the sample matrices used in this study inhibited the PCR, as shown by internal DNA standards. Internal standards consisted of 1 pg of extracted *B. pumilus* genomic DNA added to a PCR.

Nucleotide sequence accession numbers. Sequences of full-length 16S rRNA sequences generated in this study were deposited in the GenBank database under accession numbers FJ191310 to FJ194034 and GQ129843 to GQ130128.

RESULTS

Sampling and sample processing. Over a period of 18 months, 107 individual surface samples were collected from three distinct NASA SAC. Due to the limitations and challenges associated with processing samples of such extremely low biomass, several individual samples from a given sampling event/locale were pooled, which resulted in 10 distinct pooled samples for further analysis (Table 1). These pooled samples were grouped into three separate categories, based on the presence/absence of spacecraft and the mission it was supporting in a given SAC, as is shown in Table 1. Category A consisted of 37 samples that were collected from SAC in the presence of Phoenix spacecraft hardware, category B comprised 30 samples that were collected within SAC devoid of spacecraft, and category C consisted of 40 samples collected from an SAC (JPL-SAF) during the assembly of the Mars Science Laboratory (MSL) spacecraft.

Even though all 10 pooled samples yielded full-length 16S rRNA gene amplicons (1.5 kb), only seven gave rise to clone libraries following transformation of competent *E. coli* TOP 10. Despite the faint visibility of some bands in agarose gels, the PCR products from all 10 of these samples were sufficient to be analyzed via PhyloChip DNA microarray. The number of distinct OTUs detected by conventional cloning and/or PhyloChip DNA microarray analyses in each sample is given in Table 1.

Clone library-derived bacterial diversity. The number of high-quality sequence reads, number of detected OTUs, coverage values, and relative increase in detection via PhyloChip DNA microarray, per sample, are provided in Table 1. After chimeras were disregarded, 76% of the total 3,852 full-length

TABLE 1. Changes in bacterial diversity of clean rooms during various spacecraft assembly phases of two different missions

Surface type and sample	Sampling condition (date of collection)	Facility	No. of samples pooled	No. of clones sequenced	No. of high-quality sequences[a]	No. of OTUs detected once	Coverage value[b]	No. of OTUs detected by cloning	No. of OTUs detected by PhyloChip	Fold increase in OTUs by PhyloChip
Phoenix spacecraft facility surfaces										
MTF-PHX-D1	During Phoenix (October 2007)	LMA-MTF	7	672	448	15	96.7	33	1,222	37
PHSF-PHX-D1	During Phoenix (June 2007)	KSC-PHSF	10	160	134	10	92.5	18	1,172	65
PHSF-PHX-D2	During Phoenix (July 2007)	KSC-PHSF	20	768	637	39	93.9	48	1,519	32
Facility surfaces with no spacecraft										
PHSF-PHX-B	Before Phoenix (April 2007)	KSC-PHSF	10	1,152	959	129	86.5	166	1,491	9
PHSF-PHX-A	After Phoenix (August 2007)	KSC-PHSF	10	608	468	7	98.5	18	728	40
SAF-MSL-B	Before MSL (October 2007)	JPL-SAF	10	396	224	59	73.7	91	1,492	16
MSL spacecraft facility surfaces										
SAF-MSL-D1	During MSL (January 2008)	JPL-SAF	10	96	68	5	92.6	7	491	70
SAF-MSL-D2	During MSL (February 2008)	JPL-SAF	10	None[c]	0	0	0	0	924	—[d]
SAF-MSL-D3	During MSL (June 2008)	JPL-SAF	10	None	0	0	0	0	697	—
SAF-MSL-D4	During MSL (August 2008)	JPL-SAF	10	None	0	0	0	0	468	—

[a] Number of clones after eliminating low-quality reads and chimeras.
[b] See Materials and Methods for calculation of coverage values.
[c] The amplification of the 1.5-kb fragment of the 16S rRNA gene failed.
[d] Due to the absence of clones, the value could not be determined.

16S rRNA sequences generated were deemed of sufficient quality to be included in this study. For a detailed breakdown of the clones including relative abundance, members assigned to each bacterial family, or genera based on the Ribosomal Database Project classifier, see Table S1 in the supplemental material.

A trend was observed linking coverage values to the presence or absence of spacecraft hardware in the SAC. Based on clone library-derived coverage values, sampling efforts were incomplete for category B samples (those collected prior to the arrival of spacecraft hardware), as was evident in two different facilities (74% to 87%) (Table 1). However, samples falling into categories A and C, which were collected in the presence of spacecraft hardware, yielded much higher coverage values (>94%). While the sample collected after Phoenix Lander assembly/occupancy (PHSF-PHX-A; category B) may seem to contradict such a trend (98.5% coverage), it must be noted that the PHSF SAC in which this sample was collected was kept under stringent maintenance (at the same maintenance level as categories A and C) in the unlikely event of launch delay and consequential return of the Phoenix spacecraft. A similar trend was noted in the occurrence of singleton OTUs (taxa whose unique sequences are retrieved only once), suggestive of broader bacterial diversity prior to the arrival of spacecraft hardware in SAC (59 in JPL-SAF and 129 in KSC-PHSF). Of the SAC sampled, the JPL-SAF (0 to 7 OTUs) exhibited significantly less diversity than either the LMA-MTF (33 OTUs) or KSC-PHSF (18 and 48 OTUs), as assessed by 16S rRNA-based cloning (Table 1).

Perhaps the most striking of all cloning-based results was the correlation between the incidence of streptococci and the presence of humans due to MSL hardware assembly in the JPL-SAF. Upon introduction of MSL hardware, the bacterial diversity was drastically reduced to streptococci only, devoid of even the cosmopolitan *Acinetobacter*, *Bradyrhizobium*, and *Ralstonia* species (data not shown). Also evident via cloning approaches was a considerable disparity in bacterial diversity between MSL- and Phoenix-housing SAC. As Table 1 clearly illustrates, SAC involved in the housing of Phoenix hardware had significantly more diverse bacterial populations than those associated with the MSL spacecraft.

DNA microarray-derived bacterial diversity. When analyzed with PhyloChip DNA microarrays, samples obtained from one sampling trip (MTF-PHX-D1, where D1 indicates a sampling period during Phoenix Lander assembly/occupancy) to the LMA-MTF housed 1,222 distinct OTUs (Table 1). Samples obtained from four sampling events at the KSC-PHSF yielded between 728 and 1,519 OTUs, and samples collected from five samplings of the JPL-SAF yielded between 468 and 1,492 OTUs. PhyloChip DNA microarrays were able to detect the presence of anywhere from 9- to 70-fold more bacterial taxa than 16S rRNA-based cloning approaches. The PhyloChip detected biosignatures in more than 140 known bacterial families, almost 100 of which were never observed in any of the clone libraries (Fig. 1). Furthermore, meaningful data were retrieved from three samples with PhyloChip arrays even after several attempts at PCR and subsequent TA cloning regimes had failed (Table 1).

Whenever possible, the authors limited bacterial taxonomic classification to the family level for the purposes of this study. However, certain taxa were either not able to be resolved below the level of order or had previously been described as genera belonging to orders lacking familial assignments (16). Since it was not feasible to provide familial taxonomic description to such taxa, in these instances OTUs were categorized according to their first available classified taxonomic levels. Along these lines, there were 107 bacterial lineages (total, 280) that simply could not be included for consideration at the family level due to the degenerate nature of the PhyloChip taxonomic calling procedure. For example, PhyloChip probes specific for phylum, class, and order that lacked specific probes for family level resolution or lower were removed from consideration. Furthermore, OTUs designated at the family level as "unclassified" belonged to an order, class, or phylum that is recognized by the Hugenholtz taxonomy (8, 16, 22) but contained insufficient sequence data in public databases to form a cluster below that taxonomic level.

Correlations were observed between the detected presence of certain bacterial families and the presence or absence of Phoenix or MSL hardware in SAC facilities. The PhyloChip exclusively inferred the presence of *Dictoglomaceae* and *Leuconostocaceae* members, and cloning approaches exclusively detected members of *Burkholderiaceae*, *Pseudomonodaceae*, and *Aurantimonadaceae* in SAC devoid of spacecraft (category B); but neither method was able to detect such microbes when spacecraft were present (categories A and C) (Fig. 2). Certain bacterial families appeared to be associated with a given spacecraft, as is evident in the PhyloChip-derived detection of *Actinosynnemataceae*, *Halothiobacillaceae*, *Hyphomonadaceae*, *Intrasporangiaceae*, and *Vibrionaceae* solely in samples collected in the presence of the Phoenix spacecraft (category A). Similarly, *Procabacteriaceae* spp. were uncovered via PhyloChip in the presence of either the Phoenix (category A) and/or MSL (category C) spacecraft, but were not encountered at all when such hardware was absent (category B) (Fig. 2).

There were very few bacterial families observed to be truly cosmopolitan when assessed via cloning. Of 173 families only 3 (*Flexibacteraceae*, *Oxalobacteraceae*, and *Streptococcaceae*) were detected in all SAC categories by both PhyloChip and cloning approaches (Fig. 2). There were, however, a great many bacterial families (130) whose presence was revealed in all three SAC categories exclusively by PhyloChip (Fig. 2). Species likely to be novel were encountered within 10 bacterial families as these were detected via cloning, yet their presence remained elusive to the PhyloChip. It was overly apparent that the MSL spacecraft housed a significantly more limited bacterial diversity than the Phoenix. While not a single family was encountered solely in the presence of MSL hardware, 23 families of bacteria were detected while either in the presence of Phoenix (category A) or in empty SAC (category B) but were not detected when MSL was present (category C).

Category C samples were collected from an SAC located in an arid, desert-like location (JPL, Pasadena, CA; relative humidity, 40 to 45%), whereas category A samples were collected from a brackish, swamp-like location (KSC, Cape Canaveral, FL; relative humidity, 60 to 85%). Changes in relative abundance at the OTU level, as inferred from PhyloChip DNA microarray, of category A and C samples are depicted in Fig. 3. This analysis can readily be determined between samples based on the fluorescence intensity of OTU probe sets, where a change in 500 relative fluorescence

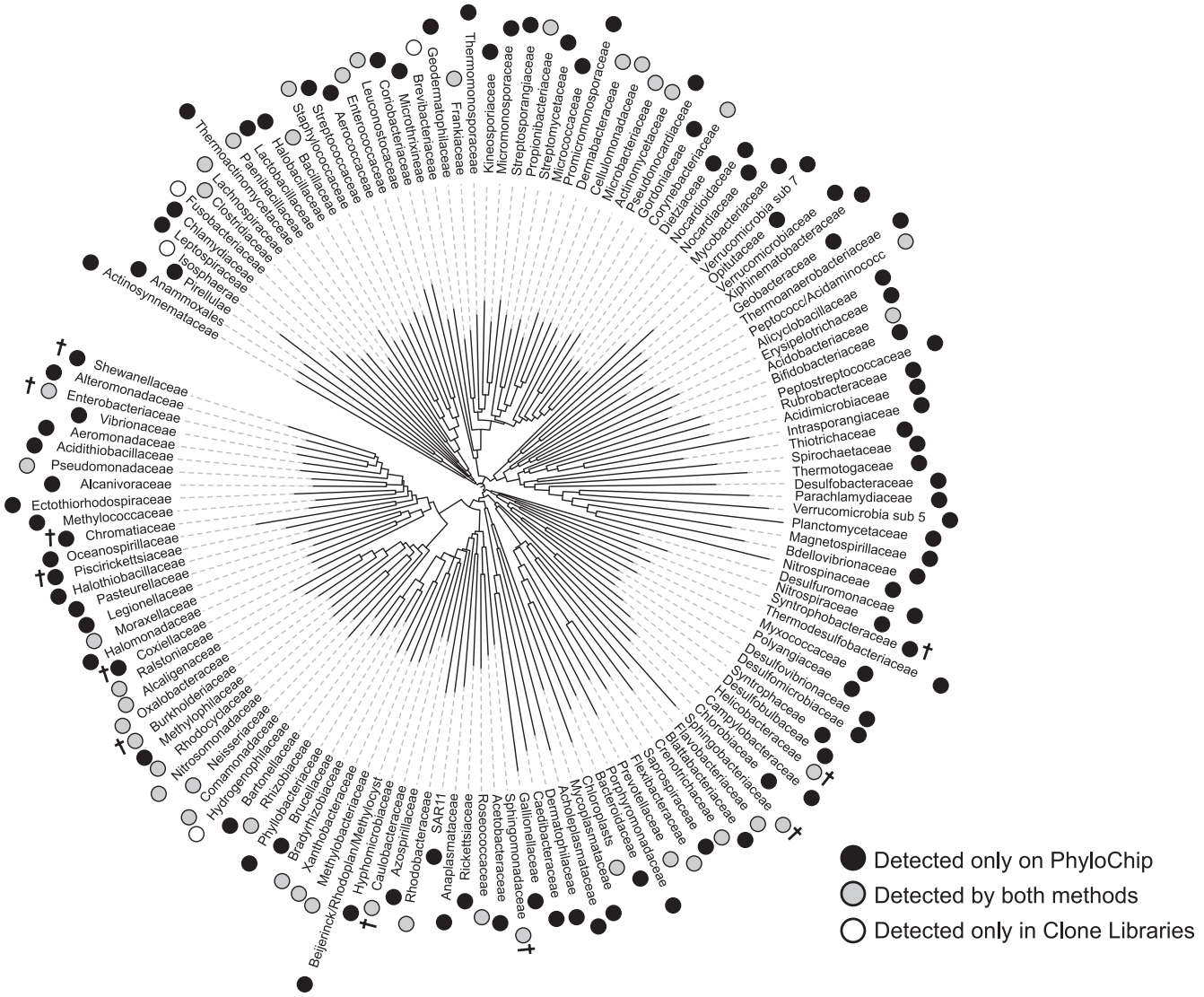

FIG. 1. Family level phylogenetic tree of bacterial taxa detected across all sampling events using PhyloChip technologies, clone library sequencing, or both. The unclassified bacterial groups (~30) were not included in the construction of this phylogenetic tree. Bacterial groups in which the PhyloChip detected significant differences in biodiversity between the JPL-SAF and the KSC-PHSF are noted (†) and described further in Table 2.

units corresponds to an approximately fivefold change in 16S rRNA gene copy number (2). Members of genera known to thrive in dry conditions exhibited high fluorescence intensities in category C samples, sensu lato, and included *Bacillus*, *Clostridium*, *Streptococcus*, and some actinobacteria, epsilonproteobacteria, and mollicutes. Conversely, alpha, beta, gamma, and deltaproteobacteria yielded significantly elevated fluorescence intensities in category A samples. An expanded, statistically significant (*P* value of >95%) biodiversity was observed in aquatic bacterial families in samples collected at the more humid KSC in comparison to those collected at JPL (Table 2).

DISCUSSION

While molecular biology has seen monumental advances in the specificity and sensitivity of modern techniques, the efficient collection and accurate phylogenetic analysis of microorganisms from low-biomass samples remain extremely challenging (19). Since the effective sampling area of a spacecraft is fixed, it is not possible simply to increase the sample size to improve yield (4). It is of utmost importance to ensure that current methods of assessing phylogenetic breadth and overall microbial burden from these precious allotments are optimal for conserving the true microbial community structure of the sampled environment. Therefore, as reported previously (19), considerable measures were taken to ensure that optimized sample collection and automated sample processing procedures were integrated so as to elucidate the fullest possible spectrum of microbial life associated with spacecraft surfaces. Given the constraints inherent to working with such low-biomass samples, technologies capable of accurately registering low-abundance organisms are vital.

The rapidity, repeatability, comprehensiveness, and sensitivity of the PhyloChip for surveying entire bacterial communities

Taxonomical position	Category A	Category B	Category C	Taxonomical position	Category A	Category B	Category C	Taxonomical position	Category A	Category B	Category C	Taxonomical position	Category A	Category B	Category C
Acidimicrobiaceae				Pasteurellaceae				Flexibacteraceae				Acholeplasmataceae			
Acidithiobacillaceae				Peptococcaceae				Oxalobacteraceae				Acidothermaceae			
Acidobacteriaceae				Phyllobacteriaceae				Streptococcaceae				Bacteroidaceae			
Actinomycetaceae				Piscirickettsiaceae								Bdellovibrionaceae			
Aerococcaceae				Planctomycetaceae				Bradyrhizobiaceae				Cardiobacteriaceae			
Aeromonadaceae				Polyangiaceae				Burkholderiaceae				Chlamydiaceae			
Alcanivoraceae				Porphyromonadaceae				Caulobacteraceae				Dietziaceae			
Alicyclobacillaceae				Promicromonosporaceae				Comamonadaceae				Entomoplasmataceae			
Alteromonadaceae				Propionibacteriaceae				Enterobacteriaceae				Francisellaceae			
Anaplasmataceae				Pseudoalteromonadaceae				Lactobacillaceae				Gordoniaceae			
Azospirillaceae				Pseudonocardiaceae				Methylobacteriaceae				Microthrixineae			
Bartonellaceae				Ralstoniaceae				Moraxellaceae				Nocardiaceae			
Bifidobacteriaceae				Rhodocyclaceae				Neisseriaceae				Prochlorales			
Blattabacteriaceae				Rickettsiaceae				Pseudomonadaceae				Roseiflexales			
Caedibacteraceae				Rikenellaceae				Sphingobacteriaceae				Streptosporangiaceae			
Caldithraceae				Roseococcaceae				Sphingomonadaceae				Syntrophomonadaceae			
Campylobacteraceae				Rubrobacteraceae				Staphylococcaceae				Thermotogaceae			
Caryophanaceae				Saccharospirillaceae				Xanthomonadaceae				Verrucomicrobiaceae			
Catabacteriaceae				Shewanellaceae								*Geitlerinema*			
Cellulomonadaceae				Simkaniaceae				Carnobacteriaceae				*Spirulina*			
Chlorobiaceae				Spirochaetaceae								AMD clone group (AF523882)			
Coriobacteriaceae				Sporichthyaceae				Acetobacteraceae				Dechlorinating clone group (AF523965)			
Corynebacteriaceae				Sporolactobacillaceae				Bacillaceae				OP8 (AF419671)			
Coxiellaceae				Streptomycetaceae				Chromatiaceae				SAR11 (AF353223)			
Crenotrichaceae				Succinivibrionaceae				Clostridiaceae				SAR86 (AF406526)			
Dermabacteraceae				Syntrophaceae				Erysipelotrichaceae				EB1021 group (AF523886)			
Dermatophilaceae				Syntrophobacteraceae				Lachnospiraceae				Ellin6075/11-25 (AY211077)			
Desulfoarculaceae				Thermoactinomycetaceae				Peptostreptococcaceae							
Desulfobacteraceae				Thermodesulfobacteriaceae				Rhodobacteraceae				Procabacteriaceae			
Desulfobulbaceae				Thermomonosporaceae								*Deferribacer*			
Desulfohalobiaceae				Thiotrichaceae				Alcaligenaceae				*Leptolyngbya*			
Desulfomicrobiaceae				Xanthobacteraceae				Brucellaceae				Uranium waste clones (AJ536882)			
Desulfovibrionaceae				Xiphinematobacteraceae				Flavobacteriaceae							
Desulfuromonaceae				*Natronoanaerobium*				Rhizobiaceae				Parachlamydiaceae			
Ectothiorhodospiraceae				*Plectonema*								LD1PA group (AY114324)			
Enterococcaceae				*Pseudanabaena*				Prevotellaceae							
Eubacteriaceae				AD3 (AJ536867)								Dictyoglomaceae			
Flammeovirgaceae				BRC1 (AY218548)				Nocardioidaceae				Leuconostocaceae			
Frankiaceae				CH21 cluster (AY222300)								*Exiguobacterium*			
Geobacteraceae				aquatic clone (AY221036)				Fusobacteriaceae				NC10 (AY177763)			
Geodermatophilaceae				BD2-10 group (AY193208)											
Halobacillaceae				DSS1 (AJ306783)				Aurantimonadaceae				Actinosynnemataceae			
Halomonadaceae				Gut clone group (AY207065)				Burkholderiaceae-5*				Halothiobacillaceae			
Helicobacteraceae				OP10 (AF524022)				Pseudomonadaceae				Hyphomonadaceae			
Hydrogenophilaceae				OP3 (AY013695)				Pseudomonadaceae-6*				Intrasporangiaceae			
Hyphomicrobiaceae				OP9 (AY013695)								Vibrionaceae			
Kineosporiaceae				SPAM (AJ519639)				Rhodospirillaceae				*Fulvimarina*			
Legionellaceae				SUP05 (AF382104)								*Phormidium*			
Legionellales				Ellin307/WD2124 (AY221039)				Clostridiaceae-11*				*Verorhodospirilla*			
Lentisphaerae				Ellin314/wr0007 (AF498715)				Deinococcaceae				OP11-5 (AF316799)			
Leptospiraceae				Ellin329/Riz1046 (AB081581)				Planococcaceae							
Magnetospirillaceae				Ellin6095/SC-I-39 (AY221080)											
Methylococcaceae				GAO cluster (AF361096)				Cyanobacteria-1*							
Methylophilaceae				KSA1 (AF449785)				Ruminococcaceae							
Microbacteriaceae				mgA-1 (AACY01094130)											
Micrococcaceae				mgA-2 (AF382142)											
Micromonosporaceae				MND1 clone group (AY221081)											
Mycobacteriaceae				NC10-1 (AY177763)											
Mycoplasmataceae				NC10-2 (AJ519650)											
Myxococcaceae				Symbiontic clone (AF432146)											
Nitrosomonadaceae				TM6 (AY043739)											
Nitrospiraceae				TM7 (337880)											
Nocardiopsaceae				TM7-3 (AY134895)											
Oceanospirillaceae				WS3 (AJ535231)											
Paenibacillaceae				WS5 (AF419661)											

Detected by:
PhyloChip
Cloning
Both
No detectable bacterial families
* Unclassifed family
Family that does not have cultivable organism (Representative GenBank # are given in parentheses)
OTUs were categorized according to their first available classified taxonomic levels

FIG. 2. Bacterial families detected across various SAC categories, as defined in the text.

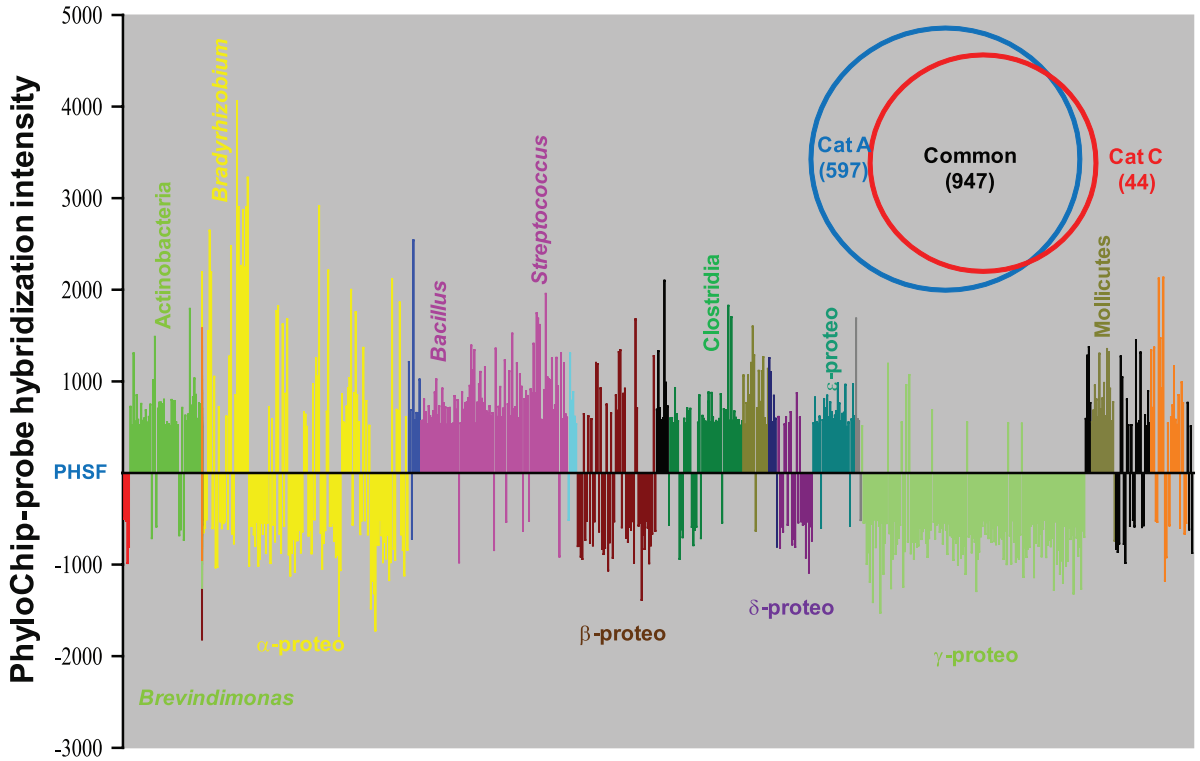

FIG. 3. PhyloChip analysis of complete bacterial communities as a function of SAC categorization. Bacteria are ordered alphabetically from left to right according to taxonomic affiliation. Bars above the zero line represent bacteria that increased in abundance relative to the JPL-SAF during the MSL mission; bars below represent those bacteria that declined in abundance. Venn diagrams demonstrate the number of bacterial subfamilies detected in each SAC category. proteo, proteobacteria.

in environmental samples suggest that the approach could significantly advance microbial detection and environmental monitoring. Key features that set the PhyloChip apart from similar technologies are the use of multiple oligonucleotide probes for all known prokaryotic taxa for high-confidence detection and the pairing of a mismatch probe for every perfectly matched probe to minimize the effect of nonspecific hybridization (35). A strong linear correlation has been confirmed between microarray probe set intensity and concentration of OTU-specific 16S rRNA gene copies, allowing quantification in a broad dynamic range. Validation experiments have demonstrated high reproducibility as intensity responses among replicate chips show less than 10% variation (3). PhyloChip results from complex environmental samples have been confirmed by additional methods, including quantitative PCR and 16S rRNA gene clone libraries (7), and analyses of split samples have confirmed that >90% of all 16S rRNA sequence types identified by the more expensive clone library method are also identified by the PhyloChip. When the high-density PhyloChip microarray, with all known DNA sequences encod-

TABLE 2. Bacterial OTU occurrence as a function of geographical sampling locations

Family[a]	No. of OTUs that can be detected by PhyloChip	No. of OTUs in clean room floors by facility type and sampling period							*P* value[b]
		PHSF			SAF				
		PHX-B	PHX-D1	PHX-A	MSL-B	MSL-D1	MSL-D2	MSL-D3	
Syntrophobacteraceae	35	8	7	8	7	4	3	2	0.04
Sphingomonadaceae	98	41	36	40	31	33	28	16	0.04
Sphingobacteriaceae	39	5	7	8	4	2	4	3	0.04
Shewanellaceae	5	5	5	5	3	1	2	0	0.01
Piscirickettsiaceae	28	4	4	4	3	2	3	1	0.04
Helicobacteraceae	64	23	21	25	22	14	11	9	0.04
Enterobacteriaceae	183	52	65	65	54	10	6	3	0.03
Coxiellaceae	15	5	4	5	3	4	2	1	0.04
Chromatiaceae	44	7	7	6	2	3	1	0	0.00
Caulobacteraceae	30	12	14	17	7	12	10	7	0.04
Burkholderiaceae	38	11	13	9	8	9	4	5	0.04

[a] Bacteria associated with aquatic environments.
[b] Differences between KSC and JPL facilities were considered significant at a *P* value of 0.05 (Student *t* test).

ing bacterial and archaeal 16S rRNA (9), was applied to urban aerosols, the spatiotemporal distributions of known bacterial groups, including specific pathogens, were related to meteorologically driven transport processes as well as sources (12).

Previous analyses of surface samples collected at three different time periods (before, during, and after Phoenix Lander assembly/occupancy) from the same locations within the KSC-PHSF clean room led to the conclusion that cleaning protocols in use were indeed effective in significantly reducing both microbial burden (13) and diversity (P. Vaishampayan et al., submitted). As might be expected, the clone libraries representing the pre-Phoenix sampling (PHSF-PHX-B, where PHX-B indicates before Phoenix Lander assembly/occupancy) exhibited a great many OTUs (166 OTUs by cloning), and the corresponding coverage value was low (86.5%). However, with increased cleaning efforts during (Table 1, PHX-D1) and after (PHX-A) Phoenix, detectable OTUs were significantly reduced (~20), and coverage values escalated to ~92%. Such a trend was also observed with samples collected before (MSL-B; 76% coverage), and during (MSL-D1; 93% coverage) MSL occupancy of the JPL-SAF. These observed reductions in bacterial numbers while facilities were housing spacecraft (Table 1, coverage values) can likely be attributed to more diligent cleaning efforts as the frequency of cleaning increased (two- to threefold increase in schedule) when spacecraft were present, as opposed to standard facility maintenance during nonoperational periods (twice per week). Immediately following the departure of the Phoenix spacecraft from the KSC-PHSF, the facility was maintained at utmost stringency, and no changes were made in cleaning practices so that the facility would be ready to accommodate any unforeseen needs associated with a launch delay. Samples collected at this time (post-Phoenix with bolstered cleaning and maintenance) continued to exhibit appreciable coverage values (98.5%) even though the spacecraft was not present.

Perhaps the greatest advantage of cloning-based biodiversity analysis was the ability to generate rarefaction curves and corresponding coverage values, which provided an invaluable approximation of just how representative each sample was of its true environment (31, 32). Due in large part to biases in the generation and picking of transformant colonies, PhyloChip DNA microarrays detected a much broader biodiversity than clone libraries, even at very high taxonomic levels (7, 35). There was an appreciable difference in the level at which the PhyloChip "out-detected" cloning approaches, based on the presence or absence of spacecraft hardware at the time of sample collection. The superior detection capabilities of the PhyloChip were far more pronounced when the facility was sampled while housing spacecraft hardware (32- to 70-fold) than when sampled facilities sat vacant (9- to 16-fold). This was a reasonable correlation since the bacterial diversity associated with any given SAC should be a combination of the bacterial diversities associated with that facility plus that associated with foreign spacecraft components that have been fabricated from countless geographic locations.

Compared directly, MSL-supporting SAC samples did not house as rich a diversity of bacteria as samples collected from facilities housing Phoenix hardware. This is not to say that MSL-associated SAC were not diverse. DNA microarray analyses detected roughly 4,000 OTUs in the five MSL-associated SAC samples; however, only ca. 150 OTUs were detected in all five of these samples. This is of immense consequence for planetary protection and/or the validation of clean room maintenance as it suggests that frequent monitoring is required over the course of a project or process to confidently assess the majority of contaminant microbes associated with production/assembly facility surfaces (and therefore at risk of being sent into space on spacecraft).

The systematic approach taken during this study revealed that the PhyloChip microarray analyses were superior to conventional 16S rRNA gene cloning and sequencing strategies in all aspects of microbial diversity analysis save one: the detection of novel microbial taxa. Since DNA microarrays are dependent on the hybridization of environmental oligonucleotides to known probes of specific sequence, an enormous amount of a priori sequence information is required. This need for previously inferred probe sequence data precludes the ability of this technique to detect the presence of DNA arising from novel microorganisms. As shown in Fig. 1, there were a few novel taxa whose presence completely eluded the PhyloChip but was inferred from clone library analysis alone. As for limitations, with the cloning and mass sequencing approach there was likely a molecular bias that favored the PCR amplification and/or amplicon ligation of certain bacterial lineages and hence masked the detection of taxa that were present in much lower abundance. High-throughput approaches possess a significant advantage to cloning in that they are much more capable of yielding valuable phylogenetic information from samples (7). Ultimately, PhyloChip DNA microarray analyses supported, and accentuated, the general trends observed by clone libraries with regard to geographic clustering (data not shown). The results of this comparative study underscore a central theme in current molecular biology: a shift toward high-throughput, data-rich molecular assays requiring significant bioinformatics analysis.

There are numerous factors to consider in choosing an appropriate methodology for elucidating microbial diversity in environmental samples. While factors of cost, time, labor intensity, and reproducibility weigh quite heavily individually, the bias and accuracy of a given approach are perhaps the most important aspects in considering the goal of planetary protection endeavors. In an effort to significantly strengthen the inferences drawn from extraterrestrial life detection experiments, NASA has stressed the importance of taking necessary precautions to ensure that spacecraft outbound from Earth are as devoid of microbial contaminants as reasonably possible. One approach to achieving this objective is to routinely survey and catalog the genetic microbial inventory present on SAC and colocated spacecraft surfaces. These efforts will prove invaluable in interpreting the findings of numerous robotic extraterrestrial life detection missions. By working to minimize the microbial burden associated with robotic spacecraft to levels approaching near sterility and routinely sampling from and maintaining a genetic inventory of the microbes associated with spacecraft and SAC, planetary protection efforts are (i) minimizing the likelihood that life detection experiments will be compromised by contaminant terrestrial biomatter, (ii) increasing the ability to discriminate authigenic from contaminant biomaterial should any be detected, and (ii) benefitting a wide range of scientific, electronic, homeland security, medical,

and pharmaceutical ventures by developing superior means of detecting and mitigating microbial contaminants from low-biomass environments.

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